Dynamic evaluation of mesenchymal circulating tumor cells in patients with colorectal cancer: Clinical associations and prognostic value

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Abstract. Circulating tumor cells (CTCs), as the precursor of metastases, gain mesenchymal traits through the epithelial-mesenchymal transition (EMT) process, thereby mediating tumor metastasis. However, the dynamic changes and clinical value of mesenchymal CTCs (^MCTCs) in colorectal cancer (CRC) patients remain inconclusive. The aim of the present study was to explore the prognostic value of dynamic changes of ^MCTCs in CRC patients using our previously developed CTCBIOPSY[®] device with an immunocytochemistry assay. The results revealed that 74 out of 175 patients were pre-^MCTCs-positive and 41 out of 127 patients were post-^MCTCs-positive. Dynamical monitoring revealed that the status of ^MCTCs remained dynamically changed under the pressure of anticancer therapy, and these dynamic changes were significantly

associated with lymphovascular invasion ($P<0.001$) and TNM stage ($P=0.033$). Moreover, Kaplan-Meier survival analyses revealed that the median recurrence-free survival (RFS) and overall survival (OS) were significantly different between four groups (pre-^MCTC⁻→post-^MCTC⁻; pre-^MCTC⁻→post-^MCTC⁺; pre-^MCTC⁺→post-^MCTC⁻; pre-^MCTC⁺→post-^MCTC⁺), and patients with pre-^MCTCs⁺→post-^MCTCs⁺ had a significant shorter RFS ($P=0.001$) and OS ($P<0.001$) than the others. Univariate and multivariate Cox regression analyses demonstrated that persistent positivity of ^MCTCs before and after anticancer therapy was an independent risk factor affecting the RFS (HR: 1.302, 95%CI: 1.033-1.639, $P=0.025$) and OS (HR: 1.366, 95%CI: 1.070-1.742, $P=0.012$) of CRC patients. Collectively, these findings provided the evidence that the dynamic change of ^MCTCs during anticancer therapy can be a useful prognostic tool in CRC, indicating the important value of molecular profiling of CTCs-EMT traits in cancer management.

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Abbreviations: CTCs, circulating tumor cells; CRC, colorectal cancer; MCTCs, mesenchymal circulating tumor cells; EMT, epithelial-mesenchymal transition; ICC, immunocytochemistry; LVI, lymphovascular invasion; PNI, perineural invasion; LNM, lymph node metastasis; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; RNA-ISH, RNA *in situ* hybridization; FDA, US Food and Drug Administration; IRB, Institutional Review Board; HR, hazard ratio; CI, confidence interval; RFS, recurrence-free survival; PFS, progression-free survival; OS, overall survival

Key words: colorectal cancer, circulating tumor cells, epithelial-mesenchymal transition, dynamic change, prognosis

Introduction

Circulating tumor cells (CTCs) are currently considered as the precursor of tumor metastases, which are closely associated with the distant metastasis of human cancers (1). Currently, CTC detection is proposed as a 'liquid biopsy' approach for real-time monitoring of tumor progression (2). Given its easy accessibility via blood draw, CTCs may represent an ideal source of biomarkers for diagnosis and evaluation of the therapeutic effect compared with various imaging approaches or more invasive tissue-based biopsies. To date, numerous studies including ours have observed that CTC counts were significantly associated with multiple unfavourable clinicopathological features and dismal prognosis (3-6). In addition, we found that CTC count alone was not enough to demonstrate the important value of CTCs in metastasis, nor can it clarify the clinical value of CTC detection in cancer. Moreover, our research group as well as others demonstrated that CTCs exhibited heterogeneous characteristics at different time-points, which meant they can change in both terms of number and phenotype during anticancer treatment (7-10).

Previously, great progress has been made in elucidating the clinical significance of CTC counts and their dynamic change (6,10), however, less attention is paid to the phenotype change of CTCs during anticancer therapy. Thus, it is critical to gain insight into the phenotypic characterization of CTCs at different treatment points, which may contribute to the further understanding of CTC-mediated metastasis and improve cancer management.

The epithelial-mesenchymal transition (EMT) process, which is known to increase cell motility and invasive ability, has been identified as a crucial driver of cancer metastasis (11,12). Currently, emerging and accumulating evidence has suggested that EMT plays an important role in CTC-mediated metastasis (13), which also was demonstrated in our recent studies (14-16). CTCs acquire mesenchymal traits through EMT, thereby gaining survival advantages and improved ability to metastasize (17). Clinically, CTCs were reported to undergo EMT at different time-points during the whole procedure of anticancer therapy, including chemotherapy, radiotherapy or molecular targeted therapy (18,19). Mesenchymal CTCs (^MCTCs), characterised by suppression of E-cadherin and upregulated expression of mesenchymal markers (such as vimentin and N-cadherin), were revealed to be significantly related with drug resistance, cancer metastasis and dismal prognosis (9,18-20). Despite this, the dynamic change and prognostic value of ^MCTCs exhibited inconsistencies in various CTC detection methods. Therefore, it is essential to further explore the clinical value of the dynamic changes of ^MCTCs.

Previously, our group as well as others reported numerous methods for CTC isolation and identification that achieved efficient capture of CTCs based on their physicochemical features or antigen expression (21-24). However, these antigen-dependent approaches exhibited a narrow spectrum of CTC capture and failed to isolate the EMT-CTC subpopulations (25,26). Recently, our group and collaborators developed an optimized one-stop CTC device-CTCBIOPSY® (Wuhan YZY Medical Science and Technology Co., Ltd., Wuhan, China), which has been demonstrated to automatically and specifically isolate and identify CTCs in a label-free, quick and low-cost manner (27). Notably, this size-based method could efficiently capture EMT-CTCs from the blood of patients (28), providing great advantage for dynamic monitoring of the changes of ^MCTCs during anticancer treatment. However, the clinical and prognostic value of the dynamic changes of ^MCTCs based on this method in colorectal cancer (CRC) still require further investigation.

In the present study, the CTCBIOPSY® device was used with an immunocytochemistry assay to dynamically monitor the changes of ^MCTCs before curative resection and after adjuvant chemotherapy, and further analyzed the clinical significance and prognostic value of the dynamic changes of ^MCTCs in CRC patients.

Materials and methods

Study design and patient recruitment. This study was designed as a prospective cohort study at a single institute. From January 2014 to May 2015, consecutive CRC patients with stage II-III, treated at Zhongnan Hospital of Wuhan University were prospectively recruited in the present study.

The inclusion criteria were as follows: i) pathology confirmed as CRC; ii) underwent radical surgery; iii) received standard 6-cycle adjuvant chemotherapy (5-fluorouracil-based combination chemotherapy); iv) adequate and available medical records. The exclusion criteria were as follows: i) pre-op chemotherapy or radiotherapy; ii) patients that did not require post-operative adjuvant therapy; iii) lack of adequate CTC data; iv) succumbed after/following 30 days of surgery. After screening according to inclusion and exclusion criteria, 175 patients [114 males, 61 females; median age=62 (37-81) years] and 127 patients [77 males, 50 females; median age=61 (37-81) years] were enrolled for pre-^MCTC and post-^MCTC analyses, respectively.

All clinicopathological data of included patients were collected from the electronic medical record system. The post-operative pathological stage of CRC was performed according to the 7th edition of the AJCC/UICC tumor-node-metastasis (TNM) staging manual (29). The present study was approved by the Institutional Review Board (IRB) of Zhongnan Hospital of Wuhan University in adherence with the Declaration of Helsinki, and written informed consent was obtained from all included patients.

CTC isolation and identification. A volume of 5 ml peripheral blood samples from all included patients were collected in EDTA-containing tubes (BD Biosciences) before surgery and after 6-cycle adjuvant chemotherapy. CTCs were isolated by using our previously reported CTCBIOPSY® device and stained by Wright's staining (27). After isolation, based on the criteria proposed by other researchers (28,30,31) and reported by our previous study (27), all candidate CTCs were reviewed and identified independently by two senior cyto-pathologists who were blinded from the patients' information, and any discrepancies were resolved by discussion or consulting a third senior pathologist. The reference threshold for this device was 1 CTC, that is, 0 CTC indicated CTC negative while ≥ 1 CTC indicated positive.

CTC immunocytochemical staining. To characterize the EMT traits of CTCs, immunocytochemical staining was used as previously described (32). The antibodies, including anti-E-cadherin antibody (1:100; product code ab40772) and anti-vimentin antibody (1:100; product code ab8978; both from Abcam), were selected to respectively label either the epithelial phenotype or mesenchymal phenotype according to the manufacturer's instructions. The HCT116 and Panc-1 cell lines were used as positive controls. As a negative control for immunocytochemistry (ICC) to ensure the exclusion of cross-reactivity, the same cell lines were used with omission of the primary antibody. Evaluation of immunostaining was manually performed using the research system fluorescence microscope (BX51; Olympus Corporation). Pre-treatment mesenchymal CTCs (pre-^MCTCs) were defined as CTCs that were vimentin-positive prior to surgery. Post-treatment mesenchymal CTCs (post-^MCTCs) were defined as CTCs that were vimentin-positive at the end of 6 cycles of adjuvant chemotherapy. The reference threshold of ^MCTCs was 1 ^MCTC, and 0 ^MCTC indicated negative while ≥ 1 ^MCTC indicated positive.

Adjuvant treatment and surveillance strategy. Adjuvant treatments [including chemotherapy (5-fluorouracil-based

combination chemotherapy) and radiotherapy] and surveillance strategy were planned based on the guidelines that were recommended by the National Comprehensive Cancer Network (NCCN) for CRC clinical management. Follow-up data were available from patient files or by telephone interview with the patients or guardians. All patients were monitored either until June 1 2019 or their death. Recurrence-free survival (RFS) was defined as the time interval from the date of resection to the date of tumor recurrence or distant metastasis. Overall survival (OS) was defined as the time in months between the operation and death or last follow-up.

Statistical analysis. All statistical analyses were performed with SPSS 22.0 (IBM Corp.). Continuous variables were presented as the median with ranges (minimum and maximum) or the mean $\pm$ standard deviation (mean $\pm$ SD), and compared using the Student's t-test. The relationships between the status and dynamic changes of pre- and post-^MCTCs and clinicopathologic characteristics were analyzed with the Pearson χ^2 -test or Fisher's exact test. The cumulative probability and differences of RFS and OS among groups were determined using Kaplan-Meier curves with log-rank tests. Univariate and multivariate Cox regression analysis were further performed to identify the prognostic factors of CRC patients and estimate the corresponding hazard ratio (HR) with 95% confidence intervals (CIs). For all tests, a two-sided P-value <0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics and cohort design. A total of 248 CRC patients were preliminarily recruited into this prospective study (Fig. 1). After screening according to inclusion and exclusion criteria, 175 patients [114 males, 61 females; median age=62 (37-81) years] and 127 patients [77 males, 50 females; median age=61 (37-81) years] were finally enrolled for pre-^MCTC and post-^MCTC analyses, respectively. Among the included patients for post-^MCTC analyses, there were 58 patients (45.7%) with colon cancer and 69 patients (54.3%) with rectum cancer; tumor grade with low and middle and high (33) were 76 (59.8%) and 51 (40.2%), respectively; patients for stage II and III were 39 (30.7%) and 88 (69.3%), respectively. The detailed clinicopathologic characteristics of the included patients for pre-^MCTC and post-^MCTC analyses were respectively summarized in Tables SI and I.

According to the pre- and post-^MCTC status, included CRC patients were grouped into four cohorts: Cohort I, 54 patients whose pre- and post-^MCTCs were both negative (pre-^MCTC $\rightarrow$ post-^MCTC $^-$); cohort II, 18 patients with pre-^MCTC-negative but post-^MCTC-positive (pre-^MCTC $^-$ $\rightarrow$ post-^MCTC $^+$); cohort III, 32 patients with pre-^MCTC-positive but post-^MCTC-negative (pre-^MCTC $^+$ $\rightarrow$ post-^MCTC $^-$); cohort IV, 23 patients with pre- and post-^MCTCs both positive (pre-^MCTC $^+$ $\rightarrow$ post-^MCTC $^+$). The detailed information of the aforementioned cohorts is outlined in Fig. 1.

EMT characterization of CTCs from patients with CRC. To characterize the EMT traits of CTCs, CTCs from the peripheral blood of patients were isolated using the CTCBIOPSY®

device and stained by Wright's staining. Then, candidate CTCs were identified by morphological analysis, and cells with an abnormal morphology and an irregular nucleus, a diameter $>15\ \mu\text{m}$, a nucleus-to-cytoplasm ratio >0.8 and a hyperchromatic nucleus and nonhomogeneous staining were considered as CTCs. The representative CTC images from two included patients are presented in Fig. 2A. Furthermore, identified CTCs were stained for E-cadherin and vimentin by ICC to distinguish their EMT traits. As revealed in Fig. 2A, CTCs with E-cadherin expression were presented as brown and considered epithelial CTCs (^ECTCs), while CTCs with vimentin expression were presented as red and considered ^MCTCs.

Overall, pre-^MCTCs were detected from 74 patients with a positive rate of 42.29% (74/175) and an average count of 2.17 (Fig. 2B), while post-^MCTCs were detected from 41 patients with a positive rate of 32.28% (41/127) and an average count of 2.66 (Fig. 2C). In the stratified analysis of TNM staging, both the number of pre-^MCTCs and post-^MCTCs were significantly higher in stage III patients than that in stage II patients ($P<0.001$, respectively; Fig. 2D and E). Furthermore, the dynamic changes of pre- and post-^MCTCs under the pressure of anticancer therapy were determined, and the results revealed that 54 patients (75.00%) remained post-^MCTC-negative while 18 patients (25.00%) converted to post-^MCTC-positive among the 72 patients with pre-^MCTC-negative; by contrast, 32 patients (58.18%) converted to post-^MCTC-negative while 23 patients (41.82%) remained post-^MCTC-positive among the 55 patients with pre-^MCTC-positive (Fig. 2F).

Association of ^MCTCs with clinicopathological characteristics of CRC patients. The association of pre- and post-^MCTC status with clinicopathological characteristics of CRC patients are presented in Table SI. Pre-^MCTC-positive was significantly correlated with tumor grade ($\chi^2=6.897$, $P=0.009$), lymphovascular invasion (LVI) ($\chi^2=15.495$, $P<0.001$), tumor invasion ($\chi^2=24.044$, $P<0.001$) and lymph node metastasis (LNM) ($\chi^2=10.382$, $P=0.001$), whereas no significant association was found between pre-^MCTCs and gender, age, tumor location, tumor size, perineural invasion (PNI), tumor-node-metastasis (TNM) stage and carcinoembryonic antigen (CEA) level ($P>0.05$ for all). Post-^MCTC-positive was significantly associated with LVI ($\chi^2=8.791$, $P=0.003$) and LNM ($\chi^2=4.517$, $P=0.034$), but not related to gender, age, tumor location, tumor size, PNI, tumor invasion, TNM stage, and serum CEA level ($P>0.05$ for all).

Furthermore, the clinical associations of the dynamic changes of pre- and post-^MCTCs in CRC patients were explored, and the results revealed that both pre- and post-^MCTC-positive were significantly related with LVI ($\chi^2=22.737$, $P<0.001$) and TNM stage ($\chi^2=8.632$, $P=0.033$), but not statistically associated with gender, age, tumor location, tumor size, tumor grade, PNI, tumor invasion, LNM and serum CEA level ($P>0.05$ for all) (Table I).

Prognostic value of pre-^MCTCs and post-^MCTCs in patients with CRC. Moreover, the prognostic value of pre-^MCTCs and post-^MCTCs in patients with CRC was analyzed, and it was revealed that patients with pre-^MCTC-positive had a significantly unfavourable RFS ($P=0.002$, Fig. 3A) and OS

Figure 1. Flowchart of the prospective cohort study design. CRC, colorectal cancer; ^MCTCs, mesenchymal circulating tumor cells.

($P=0.005$, Fig. 3B) compared to pre-^MCTC-negative patients. In addition, post-^MCTC-positive patients also had a worse RFS ($P<0.001$, Fig. 3C) and OS ($P<0.001$, Fig. 3D) compared to post-^MCTC-negative ones.

For the dynamic change of pre- and post-^MCTCs, survival was significantly different among the four groups. Median RFS for patients with pre-^MCTC⁻→post-^MCTC⁻, pre-^MCTC⁻→post-^MCTC⁺, pre-^MCTC⁺→post-^MCTC⁻ and pre-^MCTC⁺→post-^MCTC⁺ were, respectively, 48.6 months, 39.2 months, 44.3 months and 32.8 months ($P=0.001$, Fig. 4A). The median OS for these four groups was, respectively, 53.2 months, 44.9 months, 50.3 months and 39.0 months ($P<0.001$, Fig. 4B). In the TNM stage-stratification analyses, the results revealed that the RFS and OS of the aforementioned four groups were not significantly different in patients with stage II (RFS: $P=0.350$, Fig. 4C; OS: $P=0.274$, Fig. 4D). For patients with stage III disease, patients with pre-^MCTC⁺→post-^MCTC⁺ had a significant shorter RFS and OS than the other three groups (RFS: $P=0.001$, Fig. 4E; OS: $P<0.001$, Fig. 4F).

Furthermore, univariate analyses indicated that poor tumor grade ($P=0.025$), presence of LVI ($P=0.001$), presence of PNI ($P=0.034$), deeper tumor invasion ($P=0.027$), more LNM ($P=0.013$), higher TNM stage ($P<0.001$) and pre-^MCTC⁺→post-^MCTC⁺ ($P=0.001$) were significantly associated with dismal RFS, while, poor tumor grade ($P=0.042$), presence of LVI ($P=0.006$), presence of PNI ($P=0.022$), deeper of tumor invasion ($P=0.042$), more LNM ($P=0.021$), higher TNM stage ($P<0.001$) and pre-^MCTC⁺→post-^MCTC⁺ ($P<0.001$) were significantly associated with unfavorable OS (Table II). Multivariate Cox regression model demonstrated that more

LNM (HR: 1.257, 95%CI: 1.052-1.751, $P=0.019$), higher TNM stage (HR: 1.794, 95%CI: 1.113-3.124, $P=0.012$) and pre-^MCTC⁺→post-^MCTC⁺ (HR: 1.302, 95%CI: 1.033-1.639, $P=0.025$) were the independent prognostic factors for shorter RFS; more LNM (HR: 1.915, 95%CI: 1.004-3.652, $P=0.049$), higher TNM stage (HR: 1.491, 95%CI: 1.138-1.955, $P=0.004$) and pre-^MCTC⁺→post-^MCTC⁺ (HR: 1.366, 95%CI: 1.070-1.742, $P=0.012$) were the independent prognostic factors for shorter OS (Table III).

Discussion

In the present study, it was revealed that the status of ^MCTCs remained dynamically changed under the pressure of anticancer therapy, and these changes were significantly correlated with multiple unfavourable clinicopathological features and dismal prognosis of patients with CRC. Furthermore, univariate and multivariate analyses demonstrated that persistent positivity of ^MCTCs before and after anticancer therapy was an independent risk factor affecting the RFS and OS of CRC patients. To the best of our knowledge, the present study is the first investigation of the potential prognostic value of the dynamic change of ^MCTCs during anticancer therapy in CRC.

Given that EMT and CTCs are the critical mediators in tumor metastasis, analyzing EMT-CTCs has great potential for cancer prognosis and progression monitoring (11,34,35). To date, more attention has been paid in exploring the clinical significance of CTC enumeration in CRC (6,7,10,36-38), and few studies have focused on the molecular traits of CTCs and its dynamic changes under the pressure of anticancer therapy.

Table I. Relationships between the change of pre- and post-MCTC status and clinicopathological characteristics of CRC patients.

Parameters	N (%)	Pre- ^M CTCs→Post- ^M CTCs				χ^2 -value	P-value
		N→N	N→P	P→N	P→P		
Sex						0.339	0.953
Male	77 (60.6)	34	10	19	14		
Female	50 (39.4)	20	8	13	9		
Age						5.661	0.129
<60 years	48 (37.8)	19	11	12	6		
≥60 years	79 (62.2)	35	7	20	17		
Tumor location						3.764	0.288
Colon	58 (45.7)	24	9	18	7		
Rectal	69 (54.3)	30	9	14	16		
Tumor size						4.740	0.192
<5 cm	47 (37.0)	29	7	6	5		
≥5 cm	80 (63.0)	51	11	26	18		
Tumor grade						3.262	0.353
Low	76 (59.8)	29	12	18	17		
Middle and High	51 (40.2)	25	6	14	6		
LVI						22.737	<0.001 ^a
Absence	82 (64.6)	45	6	22	9		
Presence	45 (35.4)	9	12	10	14		
PNI						5.821	0.121
Absence	86 (67.7)	41	9	23	13		
Presence	41 (32.3)	13	9	9	10		
Tumor invasion						1.424	0.746
T1-2	22 (17.3)	8	4	7	3		
T3-4	105 (82.7)	46	14	25	20		
LNM						1.606	0.675
N0-1	41 (32.3)	17	5	13	6		
N2-3	86 (67.7)	37	13	19	17		
TNM stage						8.632	0.033 ^{a,b}
II	39 (30.7)	16	3	16	4		
III	88 (69.3)	38	15	16	19		
CEA level						6.945	0.076
<5 ng/ml	87 (68.5)	35	9	27	16		
≥5 ng/ml	40 (31.5)	19	9	5	7		
Overall	175 (100.0)	54	18	32	23	-	-

^aP<0.05. ^bFisher's exact test. CRC, colorectal cancer; ^MCTCs, mesenchymal circulating tumor cells; N→N, negative→negative; N→P, negative→positive; P→N, positive→negative; P→P, positive→positive; LVI, lymphovascular invasion; PNI, perineural invasion; LNM, lymph node metastasis; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen.

Zhang *et al* detected the phenotype of CTCs in advanced CRC patients during chemotherapy using a size-based platform combined with immunofluorescence staining, and the results revealed that patients with vimentin-positive CTCs had shorter progression-free survival (PFS) and OS than patients with vimentin-negative CTCs (28). In other types of solid tumors, Qi *et al* used an advanced CanPatrol CTC-enrichment technique and RNA *in situ* hybridization (RNA-ISH) to detect CTCs undergoing EMT in patients

with hepatocellular carcinoma, and the results revealed that ^MCTCs positive before surgery were significantly related to early recurrence, multi-intrahepatic recurrence, and lung metastasis (9). Another group of researchers used the same method to explore the dynamic changes of different phenotypic CTCs in renal cell carcinoma, and revealed that the recurrence or metastasis of RCC was uncorrelated with initial CTC counts, but probably related with the variation trend of CTCs, especially ^MCTCs (39). In addition, Markiewicz *et al*

Figure 2. Quantitative characterization of EMT-CTCs in patients with CRC by using CTCBIOPSY® device with ICC assay. (A) Representative CTCs images by Wright's staining and EMT-CTCs images by ICC method; Scale bar, 10 μ m. (B) The distribution of pre-^MCTC count in 175 patients with CRC. (C) The distribution of post-^MCTC count in 127 patients with CRC. (D and E) Difference in the number of pre-^MCTCs and post-^MCTCs between stage II and III CRC patients. (F) Dynamic changes of pre-^MCTC and post-^MCTC status before and after anticancer therapy. The data is expressed as the mean $\pm$ SD. CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; ^MCTCs, mesenchymal circulating tumor cells.

used general breast cancer markers to detect the EMT phenotype of CTCs, and revealed that ^MCTCs were characterized by the most aggressive phenotype, presence of LNM, larger tumor size and higher risk of death (40). In our present study, we used a size-based CTCBIOPSY® device combined with an immunocytochemistry (ICC) assay to dynamically monitor the changes of ^MCTCs before curative resection and after adjuvant chemotherapy. The results revealed that the status of ^MCTCs remained dynamically changed under the pressure of anticancer therapy, and these dynamic changes were significantly associated with presence of LVI, higher TNM stage, and dismal prognosis. Notably, persistent positivity of ^MCTCs before and after anticancer therapy was considered as an independent risk factor affecting the RFS and OS of

CRC patients. These results were consistent with previous results (7,9), indicating that the dynamic change of ^MCTCs during anticancer therapy is a useful prognostic tool for solid cancers and dynamically monitoring CTCs-EMT traits may provide more detailed information for cancer management.

The EMT process, characterized by epithelial cell loss of cell-cell junctions, acquisition of a migratory phenotype, and increased cellular motility, has been demonstrated to play a key role in tumor development (11,12). Currently, emerging and accumulating evidence has demonstrated that EMT is widely involved in CTC generation (20,35); in addition, CTCs may also undergo EMT changes during anticancer therapy to resist external intervention, thereby surviving in the peripheral system and eventually forming metastatic

Figure 3. Prognostic value of pre- and post-MCTCs in patients with CRC. (A and B) The difference of RFS and OS between CRC patients with pre-MCTCs. (C and D) The difference of RFS and OS between CRC patients with post-MCTCs. CRC, colorectal cancer; MCTCs, mesenchymal circulating tumor cells; RFS, recurrence-free survival; OS, overall survival.

Figure 4. Prognostic value of the dynamic changes of pre- and post-MCTCs in patients with CRC. (A and B) The difference of RFS and OS among four cohorts of patients with different pre- and post-MCTC status. (C-F) Stage-stratification analyses of the difference of RFS and OS among four cohorts of patients with different pre- and post-MCTC status. CRC, colorectal cancer; MCTCs, mesenchymal circulating tumor cells; RFS, recurrence-free survival; OS, overall survival.

Table II. Univariate analyses of factors associated with RFS and OS of CRC patients.

Factors	RFS			OS		
	HR	95% CI	P-value	HR	95% CI	P-value
Sex (male vs. female)	0.777	0.466-1.296	0.334	0.792	0.467-1.343	0.387
Age (<60 years vs. ≥60 years)	1.453	0.881-3.409	0.686	1.361	0.809-2.288	0.245
Tumor location (colon vs. rectal)	1.590	0.963-2.618	0.070	1.610	0.956-2.710	0.073
Tumor size (<5 cm vs. ≥5 cm)	1.450	0.772-2.748	0.251	1.063	0.637-1.773	0.817
Tumor grade (poor vs. moderate and well)	0.465	0.245-0.917	0.025 ^a	0.531	0.272-0.976	0.042 ^a
LVI (absence vs. presence)	3.001	1.582-5.684	0.001 ^a	2.065	1.236-3.450	0.006 ^a
PNI (absence vs. presence)	2.213	1.065-4.598	0.034 ^a	2.073	1.114-3.876	0.022 ^a
Tumor invasion (T1-2 vs. T3-4)	1.481	1.047-2.096	0.027 ^a	1.314	1.013-2.021	0.042 ^a
LNM (N0-1 vs. N2-3)	1.498	1.201-1.869	0.013 ^a	1.606	1.376-2.020	0.021 ^a
TNM stage (II vs. III)	2.725	1.577-4.710	<0.001 ^a	3.038	1.687-5.469	<0.001 ^a
CEA (<5 ng/ml vs. ≥5 ng/ml)	1.344	0.818-2.208	0.243	1.235	0.734-2.075	0.427
Change of pre- ^M CTCs to post- ^M CTCs	2.387	1.616-3.937	0.001 ^a	2.717	1.623-4.454	<0.001 ^a

^aP<0.05. RFS, recurrence-free survival; OS, overall survival; CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; LVI, lympho-vascular invasion; PNI, perineural invasion; LNM, lymph node metastasis; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; MCTCs, mesenchymal circulating tumor cells.

Table III. Multivariate analyses of factors associated with RFS and OS of CRC patients.

Factors	RFS			OS		
	HR	95% CI	P-value	HR	95% CI	P-value
Tumor grade (low vs. middle and high)	1.274	0.931-1.729	0.126	1.224	0.825-1.806	0.324
LVI (absence vs. presence)	1.382	0.789-2.422	0.258	1.377	0.762-2.490	0.289
PNI (absence vs. presence)	0.617	0.358-1.065	0.083	1.406	0.803-1.418	0.233
Tumor invasion (T1-2 vs. T3-4)	1.143	0.765-1.714	0.528	1.462	0.645-3.332	0.374
LNM (N0-1 vs. N2-3)	1.257	1.052-1.751	0.019 ^a	1.915	1.004-3.652	0.049 ^a
TNM stage (II vs. III)	1.794	1.113-3.124	0.012 ^a	1.491	1.138-1.955	0.004 ^a
Change of pre- ^M CTCs to post- ^M CTCs	1.302	1.033-1.639	0.025 ^a	1.366	1.070-1.742	0.012 ^a

^aP<0.05. RFS, recurrence-free survival; OS, overall survival; CRC, colorectal cancer; HR, hazard ratio; CI, confidence interval; LVI, lympho-vascular invasion; PNI, perineural invasion; LNM, lymph node metastasis; TNM, tumor-node-metastasis; ^MCTCs, mesenchymal circulating tumor cells.

tumors (9,17,20,41). The generation of CTCs requires several key steps, including detachment from the tumor lesion, invasion of the basal membrane, entry of vessels and survival in circulation. Specifically, EMT and related regulatory networks play important roles in this process, by increasing tumor cell invasiveness, promoting intravasation and facilitating tumor cell survival to mediate CTC generation (34,35). Theoretically, tumor lesions can release thousands of CTCs into the bloodstream every day, but only a few can survive in circulation because they may encounter strong anoikis signals and anticancer therapy. Nevertheless, under these stressful stimuli, CTCs could undergo EMT changes to form ^MCTCs that facilitate their survival by avoiding apoptosis, anoikis and senescence and promoting drug resistance (42,43). Additionally, EMT-inducing transcription factors (EMT-TFs),

such as Snail, Slug, Twist and SIP1, can protect CTCs from anoikis by interfering with the normal apoptosis cascade, resisting senescence, and/or collaborating with TrkB (44-47). These studies indicated that CTCs can undergo EMT phenotype changes during treatment to cope with external attacks including radiotherapy and chemotherapy to promote tumor metastasis, thereby affecting patient prognosis.

Given the important role of CTCs in tumor progression, great attempts have been made to develop reliable methods for detecting these cells in the past few decades (48,49), and numerous technologies based on the physical and biological properties of CTCs have been designed to isolate them, including cytometric methods (50), PCR-based assays (51,52), antibody-dependent methods (53), and size-exclusion technologies (54). Among these methods, the CellSearch™ system

(Veridex LLC) is the first and only method approved by the US Food and Drug Administration (FDA) for clinical application to detect CTCs in CRC (55). However, as an EpCAM-dependent isolation technology, this approach may fail to detect the CTCs undergoing EMT (e.g., ^MCTCs) (56). Recently, several non-EpCAM-based CTC isolation platforms have been developed to analyse CTCs with EMT, such as ISET[®] (Rarecells Diagnostics, Paris, France) (57), CanPatrol[™] (9,41) and the Vitatex CAM platform (58). In the present study, we used the self-developed CTCBIOPSY[®] device combined with an ICC assay to detect ^MCTCs. As an isolation by size of epithelial tumor cell assay, CTCBIOPSY[®] exhibited excellent performance in capturing the CTCs of patients (27). Previously, this size-based method has been demonstrated to efficiently capture EMT-CTCs from the blood samples of patients (28). Combined with our present results, we have reason to believe that CTCBIOPSY[®]-based EMT-CTC detection can provide a potential biomarker for prognosis assessment of CRC. Although, further studies are still required to evaluate the practical impact of EMT-CTC count on treatment strategy optimization.

There are several limitations in the present study. First, the limited number of patients included and the use of samples from a single center reduce the validity of this prospective cohort study. Second, since we did not evaluate the tumor load of patients during the process of conducting this study, the relationship between ^MCTC count and tumor load was not further analyzed. In fact, tumor load is an important indicator reflecting the malignant degree of a tumor, and its degree is closely related to the generation of CTCs. Therefore, evaluating the relationship between ^MCTCs and tumor load can provide important information for us to further understand the clinical significance of ^MCTCs in CRC. In the future studies, this will be focused on in depth.

In conclusion, the present study demonstrated that the count and status of ^MCTCs remained dynamically changed under the pressure of anticancer therapy, and these changes were significantly associated with multiple unfavourable clinicopathological parameters and dismal prognosis of patients with CRC. Further univariate and multivariate analyses demonstrated that persistent positivity of ^MCTCs before and after anticancer therapy was an independent risk factor affecting the OS and RFS of CRC patients. Collectively, these findings highlight the importance of the dynamic monitoring of ^MCTC change in the prognosis assessment, providing additional insights into the clinical significance of CTCs in CRC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

DDS, SYW and BX conceived and designed the study. DDS, CGY and SH conducted the experiments. DDS and CGY provided the study materials and assembled the patient samples. DDS collected and assembled the data. DDS and CGY prepared the figures and tables. DDS and CGY analyzed and interpreted the data. DDS and CGY wrote the manuscript. SYW and BX revised the manuscript. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board (IRB) of Zhongnan Hospital of Wuhan University in adherence with the Declaration of Helsinki, and written informed consent was obtained from all included patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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